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Retroviral Gene Transfer Into Cord Blood Stem/Progenitor Cells Using Purified Vector Stocks

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Cord blood (CB) progenitor/stem cells (P/SC) are ideal targets for early gene therapy in individuals prenatally diagnosed with genetic disorders. Most retroviral transduction protocols were developed using adult peripheral blood stem cells (PBSC) and bone marrow (BM). Less is known about retroviral transduction of CB P/SC. We examined how timing, multiplicity of infection (MOI), and polycations in the transduction media affect transduction efficiency. Rates of transduction were determined in recently isolated $CD34^+$ enriched CB cells and in colonies derived after various times in liquid cultures (LC). CB mononuclear cells (MNC) were separated by ficoll-hypaque centrifugation and enriched for $CD34^+$ cells. Purity was assessed by flow cytometry. Transduction were performed with clinical-grade retroviral stocks at MOIs of 1–20. Transduction was performed with fetal bovine serum (FBS) or autologous plasma, IL-3, GM-CSF, IL-6, and SCF. The retroviral vector contained LacZ and neomycin resistance (*neo*) reporter genes. Transduction was determined by X-gal stain and by PCR amplification of the reporter genes. No drug selection was used. Twenty-five experiments were done. CB volumes ranged from 35–150 ml. MNC and $CD34^+$ cell counts ranges were: $0.14–840 \times 10^6$ and $0.1–4.2 \times 10^6$, respectively. Transduction efficiency in liquid cultures ranged from 4–63%. Higher rates were seen using MOI $\geqslant$ 10, 2 μg/ml polybrene, and 10% autologous CB plasma. In colonies, transduction rates were 63 to 72% by PCR and 32% by X-gal staining. In LTC-IC derived colonies, transduction was 7% by PCR. Short incubations of $CD34^+$ CB cells with purified retroviral stocks, polybrene, and autologous sera result in high transduction rates of committed progenitors and moderately low efficiencies of transduction of LTC-IC in the absence of drug selection. Am. J. Hematol. 57:16–23, 1998. © 1998 Wiley-Liss, Inc.

Key words: gene therapy; umbilical cord blood; hematopoietic stem cell; retrovirus

INTRODUCTION

Many current efforts in gene therapy focus on hematopoietic stem cells (SC) [1–3]. Much of this emphasis is owed to advances in SC transplantation technology. The standard approach to genetic manipulation of SC has been based on the use of retroviral vectors. The technology for retroviral gene transfer into hematopoietic progenitor/stem cells has been extensively developed in culture systems [4–9]. Numerous in vivo studies have used retroviral transduction in conjunction with bone marrow transplantation in murine models [1,10–13], and in larger animal models including dogs [14–17] and Rhesus monkeys [18,19]. Results of human gene therapy clinical trials for adenosine deaminase deficiency [20–22] and gene marking in cancer patients [23–25] have also been reported.

In vitro studies using detection of vector sequences in hematopoietic colonies as a measurement of transduction

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have demonstrated efficient gene transfer [8,26,27]. In vivo studies in mice have shown high numbers of retrovirally marked cells after transplantation [10–12]. Although rates of gene transfer in larger animal models have been consistently lower [14,15,17,19], Bienzle et al. [16] showed 10–30% transduction efficiencies in dogs transplanted with marked autologous cells with no myeloablation. In human clinical trials, only limited transduction of SC was evident after autologous bone marrow transplantation (ABMT) [22,23]. Interestingly, among these trials, the highest success rate was accomplished in children undergoing ABMT for acute leukemia [25]. Because retroviral vectors require actively dividing cells for integration [28], one hypothesis contemplates that SC harvested from children have a higher proliferative rate than those harvested from adults. A logical extension to that hypothesis is that SC obtained earlier in ontogeny, i.e., from placental cord blood (CB), may be even better suited for retroviral transduction. This is supported by studies of the phenotypes and clonogenic potentials of CB progenitor cells compared to that of adult BM [29,30]. Recent efforts to transduce umbilical CB SC have shown high efficiency of retroviral-mediated gene transfer [31–35].

CB is an ideal target for gene manipulation. The collection represents no risk for the mother or the infant and CB contains SC with high proliferative capacity as demonstrated by successful restoration of hematopoiesis in clinical bone marrow transplantation (BMT) [36–39]. Thus, it could be used for early intervention by gene transfer. Neonatal correction of hemoglobinopathies, bleeding disorders, and immune deficiencies could prevent the development of serious sequelae from these diseases. Since obtaining neonatal bone marrow or PBSC is not technically feasible, CB P/SC targeted gene therapy provides a unique window of opportunity for this patient population.

We set out to determine the success rate of retroviral transduction in CB P/SC using transduction protocols that could be rapidly translated into clinical use. To that end, we used conventional transduction media in the absence of stromal layers or virus-producing cells and clinical-grade, high-titer amphotropic retroviral stocks. We did not attempt to manipulate the colonies through drug selection or other techniques that could impact application of the protocol for clinical trials. After a short exposure to vector-containing media, we studied the presence of vector sequences by biochemical methods and polymerase chain reaction (PCR) amplification. These studies were performed in cells in liquid culture and in hematopoietic colonies derived after short- and long-term liquid cultures. We determined the influence of vector multiplicity of infection (MOI), polycations, autologous plasma, and heterologous sera on transduction efficiency.

MATERIALS AND METHODS

Placental Blood Collection and Processing

All CB collections were from live births under an IRB approved protocol. CB was collected after delivery of the placenta by cannulation of the umbilical vein into a standard CPDA-1 blood collection set (Baxter, Deerfield, IL). All specimens were obtained within 8 h of further processing. Autologous plasma was separated by low speed centrifugation of CB and stored for later use. Recovered CB cells were diluted 1:4 with α medium (GIBCO BRL Life Technologies, Grand Island, NY). Mononuclear cells (MNC) were obtained from diluted CB by ficoll-hypaque (Pharmacia Fine Chemicals, Piscataway, NJ) centrifugation at 450*g*, 18°C, for 30 min [40,41]. $CD34^+$ cell enrichment was performed by double selection with a VARIO MACS magnetic cell separation system (Miltenyi Biotec Inc., Sunnyvale, CA) according to the manufacturer's specifications. The first separation used a medium-size (BS) column and the second a rare cell (RS) column.

All cell counts were obtained in duplicate with a Coulter ZBI automated cell counter (Hialeah, FL). $CD34^+$ cell purity following enrichment was measured by flow cytometry (FACScan; Becton Dickinson, San Jose, CA) using a 2-color pairing (anti-CD34* phycoerythrin with anti-CD45* fluorescein) of Becton Dickinson reagents. The proportion of $CD34^+$ cells among all cells identified by forward and side light scatter characteristics was defined as: % $CD34^+$ $CD45^+$/% $CD45^+$.

Vectors

Clinical-grade, high-titer amphotropic retroviral stocks (>2×10^7 infectious U/ml) carrying the bacterial genes β-galactosidase (EC 3.2.1.23) (LacZ) and neomycin phosphotransferase (EC 2.7.1.95) (*neo*) driven by the long terminal repeat of the vector were obtained from Chiron Viagene (San Diego, CA). High titer retrovirus expressing *Escherichia coli* β-*galactosidase* (42) (DA-βgal) was prepared by Chiron Viagene, Inc. (International patent publication nos. WO 95/10601 and WO 92/05266). Briefly, crude vector supernatants were clarified by 0.45 μm filtration, followed by concentration by tangential flow filtration and dialysis, size exclusion chromatography, and a second concentration and dialysis step if necessary. The final formulation was approximately 25 mM tromethamine, pH 7.4, 60 mM NaCl, 1 mg/ml arginine, 5 mg/ml HSA, and 50 mg/ml lactose. Vector preparations were shown to be free of replication competent retrovirus by hygromycin marker rescue assay of vector, or by *Mus dunni*/producer cell co-cultivated as described [42]. The viruses used for these studies had a titer of 1–3 × 10^8 cfu/ml. Viral stocks were shipped on dry ice and kept at −80°C for long-term storage. Once thawed, vector stocks were aliquoted in 1 × 10^6 particle

fractions for long-term storage. No stocks were subjected to more than one freeze-thaw cycle. All handling of virus was performed under laminar flow with standard infectious material handling precautions.

Transductions

After selection, $CD34^+$ enriched cells were collected by centrifugation and resuspended in liquid culture (LC) transduction media containing either 10% fetal bovine serum (FBS) (Armour Pharmaceutic Intergen Co., Purchase, NY) or 10% autologous plasma in α medium, 5 μg/ml gentamicin (GIBCO BRL Life Technologies), 25 μg/ml amphotericin B (Bristol-Myers Squibb Company, Princeton, NJ), 0.1 U/ml penicillin, 0.1 μg/ml streptomycin (GIBCO BRL Life Technologies), and 400 mg/ml L glutamine (GIBCO BRL Life Technologies). Hematopoietic growth factors (HGF) were added at the following concentrations: 50 U/ml interleukin-3 (IL-3, AMGEN, Thousand Oaks, CA), 2.0 ng/mL granulocyte-macrophage colony stimulating factor (GM-CSF, R&D Systems, Minneapolis, MN), 100 ng/mL IL-6 (kindly provided by Immunex, Seattle, WA), and 100 ng/mL stem cell factor (SCF, kindly provided by AMGEN). Either polybrene (Sigma, St. Louis, MO) 2 μg/ml [43], or protamine sulfate (Elkins-Sinn, Cherry Hill, NJ) 2 μg/ml was added to the transduction medium. Transductions were carried out in flasks (Falcon number 3013, Becton Dickinson, Lincoln Park, NJ) incubated at 37°C, 4% CO_2, high humidity. Pilot experiments (data not shown) determined the ideal cell concentration and volume for transductions as 3×10^5 cells/4 ml of media/flask. Cells were transduced with amphotropic virus at MOIs of 1, 10, and 20. For different MOIs, identical concentrations of polybrene (2 μg/ml) and autologous sera (10%) were used. Likewise, for comparisons of different serum sources identical concentrations of polybrene (2 μg/ml) and MOI (10) were used. Three virus inoculations were performed immediately upon initiation of transduction culture (0 h), and after 16 and 24 h. Forty hours after the initial infection, an aliquot of unstained cells was placed into methylcellulose cultures or transferred to long-term cultures (see Methylcellulose and Long-Term cultures Sections).

Methylcellulose Cultures (MC)

Methylcellulose cultures were performed as previously described [40,41]. One to 5×10^4 transduced or control $CD34^+$ cells or 5×10^4 cells recovered from long-term liquid cultures were placed in 0.8 ml methylcellulose (Fisher Scientific Co., Pittsburgh, PA) in α medium with 30% FBS, 1% bovine serum albumin (BSA, Cohn Fraction IV, Sigma), 10^{-4}M 2-β mercaptoethanol (Sigma), 0.1 U/ml penicillin, 0.1 μg/ml streptomycin (GIBCO), 100 ng/mL SCF, 2 U/mL erythropoietin (Epo, kindly provided by Ortho Pharmaceutical Corp, Raritan, NJ), 50 U/mL IL-3, and 2 ng/mL GM-CSF. MC cultures were incubated in Nunc 4-well culture dishes (0.3 ml/well) (Intermed, Roskilde, Denmark) at 37°C in 4% CO_2, high humidity. Colonies were assessed on days 7, 10, 14, 18, 21, and 28 after plating using a Leica (Rochester, NY) stereozoom dissecting microscope. Colony counts and LacZ activity determinations were performed on day 14 after plating.

Long-Term Cultures

To establish long-term stroma-free suspension cultures, previously published methods [41] were modified as follows. Each flask was seeded with 1 to 10×10^4 $CD34^+$ transduced or control cells in 8 ml of Myelocult H5100 medium (Stem Cell Technologies, Inc., Vancouver, BC, Canada) containing 2 ng/mL GM-CSF, 50 U/mL IL-3, 100 ng/mL IL-6, 100 ng/mL SCF, 0.1 U/mL penicillin, 0.1 μg/ml streptomycin, 5 μg/ml gentamicin, and 25 μg/mL amphotericin B. At weekly intervals, cells and medium were demidepleted. Four milliliters of fresh medium with hematopoietic growth factors and antibiotics were added to the flasks. An aliquot of cells ($1–5 \times 10^4$) was transferred to methylcellulose cultures to evaluate progenitor cells (BFU-E, CFU-GM, and long-term culture-initiating cells [LTC-IC, from long-term cultures >25 days]).

Detection of LacZ Activity

An aliquot of cells from transduction cultures was stained by supplementing the media with 150 μg/ml X-gal (Boehringer Mannheim Corp., Indianapolis, IN). The X-gal stock solution was prepared by dissolving X-gal, 11 mg/ml, in DMSO (Tera Pharmaceuticals, Inc., Buena Park, CA) and sterilized by γ radiation (1,620 cGy over 6′). Staining was evaluated 88 h after initial infection (48 h after X-gal stain). Assessment of color was by examination of cells in culture with an inverted microscope and by examination of cell spreads obtained with a standard cytospin apparatus. A 3T3 murine fibroblast cell line, which is stably transfected with a β-actin promoter/LacZ construct and expresses the bacterial gene at high level, was used as a positive control for the X-gal stain.

Progenitor-derived hematopoietic colonies in methylcellulose were stained by adding 150 μg/ml X-gal to cultures on day 14 after plating. Blue cells and colonies were scored after 48 h of exposure to X-gal. In liquid cultures, a minimum of 100 cells was counted under 400× magnification in at least 6 separate fields by inverted microscopy. In methylcellulose cultures, a minimum of 20 colonies was counted and scored under 100× magnification. Colony staining was graded from + to +++. Colonies with less than 1/3 cells staining blue were graded +, those with 1/3 to 2/3 were graded ++, and those with more than 2/3 of cells blue were graded +++. Two investigators scored and graded the colony stains blinded to each other's observations. Because low intensity (+),

background X-gal staining was visualized in isolated colonies derived from uninfected progenitors, only colonies displaying ++ or +++ staining were scored as positive.

PCR Detection of Retroviral Sequences

Colonies were visualized using a stereozoom dissecting microscope and individual hematopoietic colonies were picked under sterile conditions from the cultures using 10-μl Micropets (Drummond Scientific Co., Broomall, PA) as previously described [40]. Individual colonies were each placed in a sterile Eppendorf tube containing 10 μl of sterile H_2O. DNA was obtained from single colonies by rapid denaturation at 95°C for 5′. DNA from colonies was subjected to PCR amplification of LacZ and *neo* viral sequences with 200 pM of the following primers:

neo upstream: 5′TCCATCATGGCTGATGCAATGCGGC
neo downstream: 5′CATCTCCTGCTCGAAGTCTAGAGC
LacZ upstream: 5′CCATGATTACGGATTCACTGGC
LacZ downstream: 5′CAGTATCGGCCTCAGGAAGATCG.

A PCR amplification kit (Perkin Elmer, Norwalk, CT) was used for all amplification reactions. Forty cycles of amplification: 94°C × 1′, 64°C × 1′, and 72°C × 1′ were performed in a thermal cycler (Perkin Elmer). Ten nanograms of genomic DNA from the vector packaging cell line was used as a positive control. Negative controls included DNA from untransduced colonies and a no DNA (H_2O) specimen. A commercial 100-bp ladder (Gibco BRL, Gaithersburg, MD) was used as molecular weight marker. PCR amplification products were analyzed by agarose gel electrophoresis and stained with ethidium bromide. Southern transfer [44] and hybridization of PCR products to radiolabeled *neo* and LacZ specific probes was used to confirm agarose gel results.

RESULTS

CB Processing and CD34+ Cell Enrichment

Twenty-five CB collections were processed. The volumes and contents of the specimens used are illustrated in Table I. The range of volumes and yield of MNC and $CD34^+$ enriched cells after selection varied widely. There was a correlation between the number of recovered MNC and CB volume (Fig. 1). CB collections with volumes <50 ml had a very low MNC yield ($<100 \times 10^8$).

Effect of Polycations, Vector MOI, and FBS vs. Autologous Plasma on the Efficiency of Transduction in Liquid Cultures

Data from three studies evaluating the above variables separately are summarized in Table II. In one experiment, the addition of polybrene to transduction media resulted in a 2.3-fold greater percent transduction than the addition of protamine and a 15-fold greater percent transduction than in media alone. Preincubation of cells with protamine or polybrene during transduction did not inhibit subsequent colony growth when compared with colony growth of control cells (data not shown). All subsequent experiments were performed with polybrene in the transduction media. In a separate experiment, the transduction efficiency was increased 5-fold by increasing the MOI from 1 to 10. There was no further increase in transduction efficiency when the MOI was increased from 10 to 20. A final pilot study revealed that transduction was also better when the medium was supplemented with 10% autologous plasma compared to 10% FBS (1.6-fold increase).

TABLE I. Cord Blood Collections*

Parameter	Mean ± SD	Range
Volume (ml)	90 ± 29	35–150
MNC (10^6)	224 ± 219	0.14–840
$CD34^+$ (10^6)	1.2 ± 1.0	0.1–4.2
Column yield (%)	2.9 ± 4.4	0.15–19.6
$CD34^+$ purity (%)	35 ± 29	2–94

*Number of CB specimens = 25.

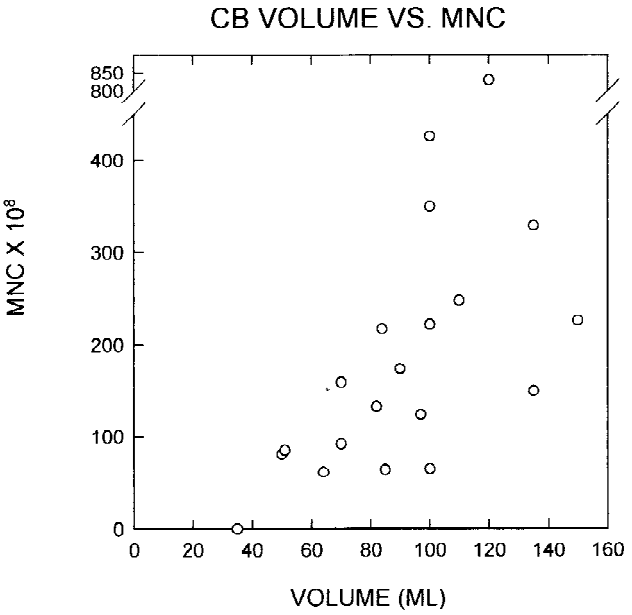

Fig. 1. X-Y plot of total MNC count yield after ficoll-hypaque separation of CB specimens in relation to CB volume.

LacZ as a Reporter Gene for the Detection of Transduction in CB Progenitor Cells

$CD34^+$ enriched cells, which were not exposed to viral vectors, did not exhibit any blue staining 24 h after ad-

TABLE II. Variables Influencing Mean Transduction Efficiency in Liquid Cultures*

	Parameter							
	Polycation			MOI			Serum source	
	None	Pr	Pb	1	10	20	FBS	AP
Mean transduction efficiency (%)	4	26	61	8	43	45	38	63

*Transduction efficiency assessment: *Neo* PCR on cfu.

dition of X-gal. Incubation with X-gal for up to 48 h led to the appearance of occasional (<1%) cells and debris that displayed blue color. In contrast, 48 h after the addition of X-gal, transduced cells developed intense blue staining (Fig. 2) and culture media displayed blue discoloration. The discoloration of the media was more pronounced in the flasks that were transduced with amphotropic vector at an MOI of 20 than an MOI of 10 (not shown).

Transduction Efficiency in CB Progenitor-Derived Hematopoietic Colonies

Individual colonies were analyzed by PCR amplification of Lac-Z and *neo* vector sequences or by staining with X-gal. A good concordance existed between transduction efficiencies estimated by amplification of the LacZ (72%) and *neo* genes (63%). A poor correlation existed between transduction efficiencies measured by X-gal stain (32%) and PCR analysis of isolated colonies (Table III).

Transduction Efficiency in Hematopoietic Colonies Derived From CB Progenitors Following Short- and Long-Term Cultures

CB $CD34^+$ enriched cells exposed to vector and uninfected controls were maintained in liquid cultures for up to 30 days. Subsequently, these cells were transferred to methylcellulose, and hematopoietic colonies that developed in semisolid media were assayed by PCR analyses for the presence of viral genes. Data are in Table IV. Similar percentages of transduced colonies were observed in hematopoietic colonies derived from cells plated immediately after transduction (48%) and those cells plated after 16 days in liquid culture (50%). A marked decrease in the rate of transduction was noted in colonies derived from cells following long-term culture for 30 days (Fig. 3).

DISCUSSION

The protocol used for CB collection in these studies is the same as that used at our institution, which is targeted for clinical transplantation. CB specimens with low volume yielded disproportionately low numbers of MNC and $CD34^+$ enriched cells. This finding is in agreement with data published by other groups [45]. For newborns prenatally diagnosed with diseases amenable to treatment by CB gene therapy, special precautions may be needed to maximize CB recovery including delivery by Caesarean section. Alternative techniques of CB collection have been proposed that result in higher volumes harvested [46]. Methods for optimizing collections without interfering with normal vaginal deliveries are needed.

Our studies demonstrate that after transduction, $CD34^+$ enriched CB cells in LC display high levels of Lac-Z activity as revealed by specific X-gal staining. This finding was not corroborated in studies of hematopoietic colonies. In uninfected colonies, non-specific staining was observed. This finding hampered the scoring of colonies as positive or negative by this method. Previous studies have demonstrated endogenous Lac-Z activity in cells of macrophage lineage, providing a possible explanation for the lack of specificity of the X-gal stain in CFU-GM [47]. Lac-Z may still prove a useful reporter gene in cells in liquid culture. However, X-gal stain may only be informative in cultures of highly purified SC as obtained by FACS sorting [31,48] or by in vitro selection with 5-fluorouracil [49].

A relatively high efficiency of transduction was verified in CB $CD34^+$ enriched cells following liquid culture and in hematopoietic colonies derived from such cells. This data is in agreement with that reported by other investigators who reported transduction efficiencies ranging between 50–80% [31–34]. However, in other studies drug selection and alternative schedules of transduction were used. Introducing chemotherapy resistance selectable genes into SC may raise some concerns for gene therapy of benign diseases.

Our results indicate that a high MOI, supplementation with autologous plasma, and the polycation polybrene facilitate transduction. This is in contrast to previous studies [50] that report similar or equally effective transduction with protamine and polybrene. We used a single concentration of polybrene (2 μg/ml) in all of our experiments. One study showed that polybrene significantly inhibits expansion of progenitors at higher concentrations [51]. It is possible that at higher doses the improved transduction efficiencies we found with polybrene may not be apparent due to higher cell toxicity. Polybrene concentrations between 2–8 mcg/ml have

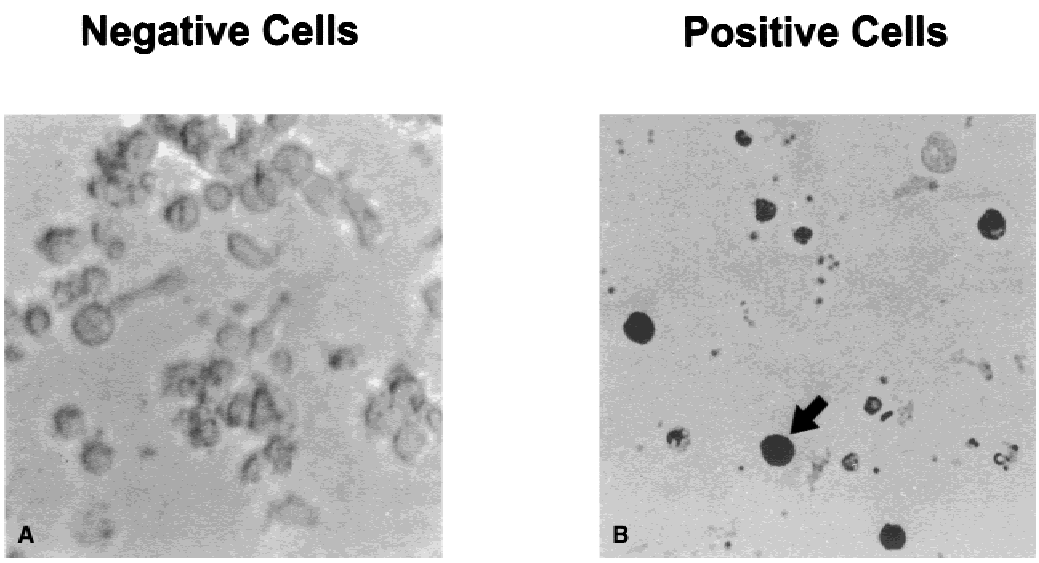

Fig. 2. Inverted microscopic view of CD34$^+$ enriched cells in LC after X-gal stain. A: Untransduced controls. B: Transduced cells. Original magnification, ×400. The arrow indicates a cell with intense X-gal stain.

TABLE III. Detection of Transduction in Progenitor Cell-Derived Colonies*

	Detection method		
	X-gal stain	LacZ PCR	*neo* PCR
Transduction efficiency (%)	32	72	63
Number of colonies assessed	515	37	46

*Transduction condition: MOI 10, polybrene 2 μg/ml, and 10% autologous serum.

TABLE IV. *Neo* PCR Assessment of Transduction Efficiency in a Representative Short- and Long-Term Culture Assay*

	Day of plating in MC from LC			
	2	16	23	30
Transduction efficiency (%)	48	50	29	7
Number of colonies assessed	222	43	31	19

*Transduction conditions: MOI 10, polybrene 2 μg/ml, and 10% autologous serum.

been used by other investigators [2,19,23,24,43,50,52]. In our studies, a low concentration was used because of concerns about toxicity to CB SC [50]. Studies are underway to examine the influence of higher concentrations of polybrene and protamine on transduction rates in CB. However, higher concentrations of polybrene may not be needed during CB SC transductions since human CB plasma, like fetal bovine serum, contains growth factors [53] that promote and support hematopoietic cell proliferation and may thus indirectly enhance transduction.

The endpoint of our studies was the detection of retroviral sequences in colonies derived from both committed and very early progenitor cells. Transduction rates in long-term culture-initiating cells (LTC-IC) may not actually correlate with transduction rates in pluripotent SC. However, we did observe a marked drop in transduction efficiencies when comparing late colonies to early colonies. This lower transduction rate in LTC-IC assays presumably reflects a smaller fraction of cells undergoing active division. This lower efficiency of transduction is seen as a limiting factor for retroviral-based gene therapy. The results presented here confirm that while high transduction efficiencies are achievable in progenitor cells, only a minority of the presumed uncommitted progenitors, as measured by the LTC-IC assay, can be infected with recombinant retroviral vectors. Clinical trials using gene marking on BM cells during recovery from chemotherapy have demonstrated stable integration of the transgene into approximately 5% of long-lived multipotent SC [25] 18 months after BM transplantation. If our LTC-IC data is predictive of the efficiency of transduction achievable in CB SC, then CB may be equivalent to BM obtained during the recovery period from intensive chemotherapy. This will ultimately need to be determined with experiments similar to those performed by Brenner et al. [25] using transduced CB SC to reconstitute autologous recipients who have had their CB specimens cryopreserved.

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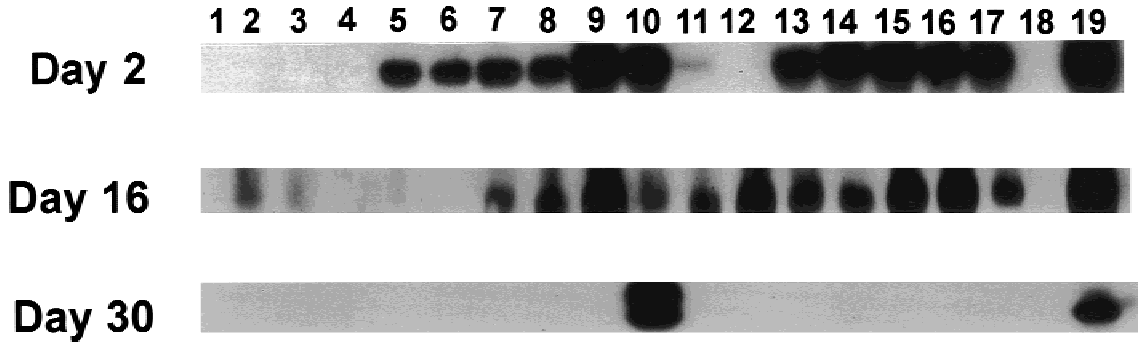

Fig. 3. Southern blot hybridization of PCR products after amplification of the *neo* gene in DNA obtained from individual colonies derived from CD34$^+$ enriched CB cells after transduction. The day on the right of each row represents the day (post-transduction) of plating in MC media. Lane 1–2, negative controls (untransduced colonies); lanes 3–17, individual colonies from one culture; lane 18, negative controls (water/no DNA control); lane 19, positive control (DNA from packaging cell line).

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